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Comments

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Protein pyrophosphorylation by inositol pyrophosphates is a posttranslational event

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In a previous study, we showed that the inositol pyrophosphate diphosphoinositol pentakisphosphate (IP₇) physiologically phosphorylates mammalian and yeast proteins. We now report that this phosphate transfer reflects pyrophosphorylation. Thus, proteins must be prephosphorylated by ATP to prime them for IP₇ phosphorylation. IP₇ phosphorylates synthetic phosphopeptides but not if their phosphates have been masked by methylation or pyrophosphorylation. Moreover, IP₇ phosphorylated peptides are more acid-labile and more resistant to phosphatases than ATP phosphorylated peptides, indicating a different type of phosphate bond. Pyrophosphorylation may represent a novel mode of signaling to proteins.

inositol polyphosphate | protein phosphorylation

Inositol phosphates have diverse biologic roles with the best known, inositol-1,4,5-trisphosphate (IP₃), releasing intracellular calcium (1). Inositol pyrophosphates (also designated inositol diphosphates), exemplified by diphosphoinositol pentakisphosphate (5-PP-I(1,2,3,4,6)P₅, hereafter called IP₇) and bis-diphosphoinositol tetrakisphosphate ([PP]₂-IP₄, IP₈) (2–4) are formed by a group of three inositol hexakisphosphate (IP₆) kinases (IP6Ks) (5–7). IP6K and inositol pyrophosphates participate in diverse physiologic activities including endocytosis (8, 9), apoptosis (10, 11), chemotaxis (12), and telomere elongation (13, 14). Recently, a novel inositol pyrophosphate, 4/6-PP-IP₅, synthesized by the Vip1 protein in *Saccharomyces cerevisiae*, was identified by York and associates (15, 16) and shown to be involved in regulating cell growth, morphology, and phosphate homeostasis in yeast.

The pyrophosphate bond of IP₇ has a calculated phosphorylation potential that equals or exceeds that of ATP, suggesting that it could serve a similar function (4, 17). We demonstrated that IP₇ physiologically phosphorylates a variety of protein targets in yeast and mammals (18). Unlike phosphorylation by ATP, IP₇ does not require separate protein kinases but directly phosphorylates its targets. We wondered how signaling by IP₇ phosphorylation is distinguished from phosphorylation by ATP. We now report that protein phosphorylation by IP₇ involves a novel modification, pyrophosphorylation, which may provide a unique mode of signaling to proteins.

Results and Discussion

In our earlier study, we showed that 5β[³²P]IP₇ phosphorylates a wide range of proteins in both the yeast, *S. cerevisiae*, and in mammalian tissues (18). We characterized in detail phosphorylation of several of these proteins, such as the yeast proteins NSR1 and SRP40. To obtain large amounts of these proteins, we expressed them in *Escherichia coli* (Fig. 1a). Whereas these proteins purified from yeast are robustly phosphorylated by 5β[³²P]IP₇, the corresponding proteins purified from bacteria display no phosphorylation. What property of yeast but not

bacterial extracts conveys the capacity for IP₇ phosphorylation? One distinction is that proteins in yeast may be prephosphorylated whereas eukaryotic proteins are unlikely to be phosphorylated in *E. coli*. We examined whether yeast extracts influence IP₇ phosphorylation of proteins purified from *E. coli* (Fig. 1b). Although yeast extracts alone do not augment IP₇ phosphorylation, yeast extracts plus ATP allow IP₇ phosphorylation of proteins purified from bacteria comparable to phosphorylation of the same proteins purified from yeast. Boiled yeast extracts fail to prime proteins for IP₇ phosphorylation, indicating that the critical factor may be a protein, possibly an enzyme (Fig. 1c).

Several lines of evidence indicate that prephosphorylation of proteins primes them for IP₇ phosphorylation. ATP and GTP, which can donate phosphate groups, provide priming activity, whereas AMP-PNP, which is nonhydrolyzable, is inactive, as is UTP, a poor substrate for protein kinases (Fig. 1d). CK2 (formerly casein kinase-2) is one of the few protein kinases for which GTP donates phosphate to a comparable extent as ATP (19). Because GTP primes IP₇ phosphorylation as well as ATP, we examined the priming activity of CK2 on the IP₇ phosphorylation of NSR1 (1–50), a fragment of NSR1 which is an excellent substrate for IP₇ phosphorylation [supporting information (SI) Fig. 5] and has a strong consensus motif for CK2 phosphorylation. CK2 robustly primes IP₇ phosphorylation (Fig. 1e). Furthermore, the IP₇ substrate NSR1 (27–50) (SI Fig. 5), can be directly phosphorylated by IP₇ when purified from *E. coli* that coexpress the catalytic subunit of CK2 (Fig. 1f). If prephosphorylation is the critical priming event for IP₇ phosphorylation, then dephosphorylation should prevent such priming. Dephosphorylation of NSR1 (51–166) or NSR1 (1–50) by λ-phosphatase greatly reduces IP₇ phosphorylation (Fig. 1g). Protein kinase-mediated priming for IP₇ phosphorylation is observed for all IP₇ substrates tested, including yeast SRP40, NSR1, YGR130c, and

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The authors declare no conflict of interest.

Abbreviations: IP₃, inositol 1,4,5-trisphosphate; IP₄, inositol tetrakisphosphate; IP₅, inositol pentakisphosphate; IP₆, inositol hexakisphosphate; IP₇, PP-IP₅, diphosphoinositol pentakisphosphate; IP₈, [PP]₂-IP₄, bis-diphosphoinositol tetrakisphosphate; IP6K, IP₆ kinase; AMP-PNP, adenylyl-5'-yl imidodiphosphate; DIPPP, diphosphoinositol polyphosphate phosphohydrolase.

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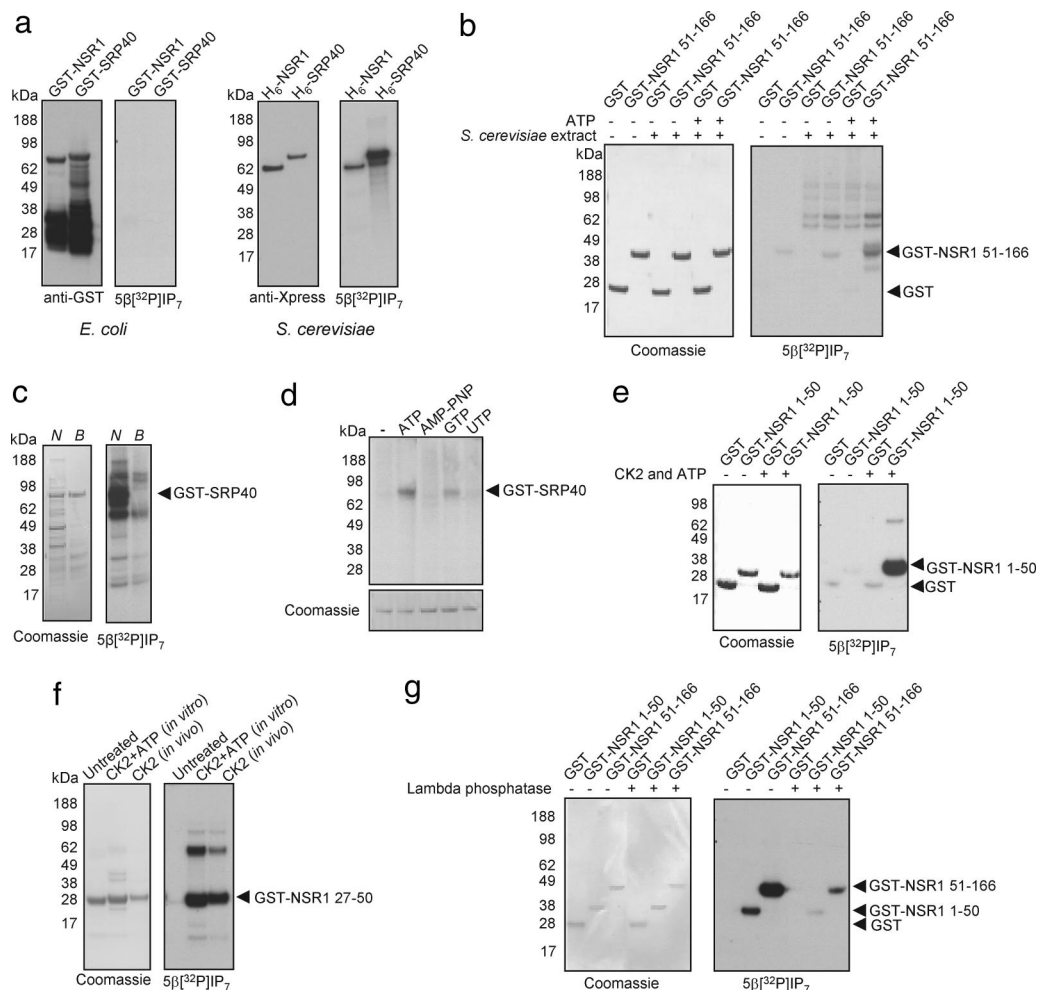


Fig. 1. IP_7 -mediated protein phosphorylation requires prephosphorylation by protein kinases. (a) NSR1 and SRP40 purified from *E. coli* or *S. cerevisiae* were incubated with $5\beta[^{32}\text{P}]\text{IP}_7$ and resolved by NuPAGE; immunoblotting with a tag-specific antibody (Left) and autoradiography to determine phosphorylation (Right). (b) GST or GST-tagged NSR1 fragment (amino acids 51–166) expressed in *E. coli* and immobilized on glutathione beads were preincubated with or without *S. cerevisiae* extract or ATP, washed with PBS, and then treated with $5\beta[^{32}\text{P}]\text{IP}_7$ and resolved by NuPAGE; Coomassie brilliant blue R250 staining (Left) and autoradiography (Right). The extra phosphorylated protein bands in lanes 3–6 are yeast proteins that bound nonspecifically to glutathione beads. (c) GST-SRP40 purified from *E. coli* was preincubated with ATP and either native *S. cerevisiae* extract (N) or extract that had been boiled for 5 min (B) and then treated with $5\beta[^{32}\text{P}]\text{IP}_7$ as in b. (d) GST-SRP40 purified from *E. coli* was preincubated with yeast extract and indicated nucleotides and then phosphorylated by $5\beta[^{32}\text{P}]\text{IP}_7$; Coomassie brilliant blue R250 staining (Lower) and autoradiography (Upper). (e) GST or GST-tagged NSR1 fragment (amino acids 1–50) purified from *E. coli* were preincubated with or without CK2 and ATP and then treated with $5\beta[^{32}\text{P}]\text{IP}_7$ as in b. (f) GST-tagged NSR1 fragment (amino acids 27–50) purified from *E. coli* was incubated without or with CK2 and ATP (lanes 1 and 2) or coexpressed in *E. coli* with the catalytic A1 subunit of human CK2 (lane 3). Purified proteins were incubated with $5\beta[^{32}\text{P}]\text{IP}_7$ as in b. (g) GST or GST-tagged NSR1 fragments (amino acids 1–50 and 51–166) purified from *S. cerevisiae*, were preincubated in the absence or presence of λ -phosphatase and then phosphorylated by $5\beta[^{32}\text{P}]\text{IP}_7$ as in b.

APL6 and mammalian Nopp140, AP3 β 3A, and TCOF1 (data not shown).

The requirement for priming by prephosphorylation suggests that either (i) ATP phosphorylation at certain sites allosterically facilitates IP_7 -mediated phosphorylation at other sites, or (ii) IP_7 directly phosphorylates amino acids that have been previously ATP-phosphorylated and hence provides a pyrophosphorylation or diphosphorylation modification. Earlier, we noted that IP_7 phosphorylation occurs primarily at stretches of serines flanked by acidic amino acids and concluded that phosphorylation occurs on the serine residues (18) (also see SI Fig. 6). In Nopp140, deletion of individual serine residues in such a stretch (amino acid 80–100) decreases IP_7 phosphorylation, implying that IP_7 can phosphorylate several of them (Fig. 2a). Substitution of the single threonine residue in this sequence has no effect on IP_7 phosphorylation. Because prephosphorylation by CK2 leads to IP_7 -mediated phosphorylation of such sites, Nopp140 fragments

containing phosphates at the CK2-preferred sites should be substrates for IP_7 phosphorylation in the absence of any other priming. To identify the preferred sites for CK2 phosphorylation, we phosphorylated an *E. coli*-expressed Nopp140 fragment comprising amino acids 80–100 and identified the major phosphorylation sites by mass spectrometry as serines 88 and 91 (data not shown). We synthesized a peptide with both of these serines prephosphorylated (Fig. 2b). This phosphopeptide is robustly phosphorylated by $5\beta[^{32}\text{P}]\text{IP}_7$, whereas the corresponding unphosphorylated peptide is resistant to $5\beta[^{32}\text{P}]\text{IP}_7$ phosphorylation (Fig. 2c). Using two peptides with phosphates at serines 88 and 91, respectively, we observe substantially greater phosphorylation at position 91 than 88 (Fig. 2c). This differential influence on discrete sites supports the specificity of IP_7 -mediated phosphorylation. Both the unphosphorylated and phosphorylated peptides are phosphorylated by $\gamma[^{32}\text{P}]\text{ATP}$ and CK2 (Fig. 2d) on one or more of the available serine residues (data not shown). IP_7

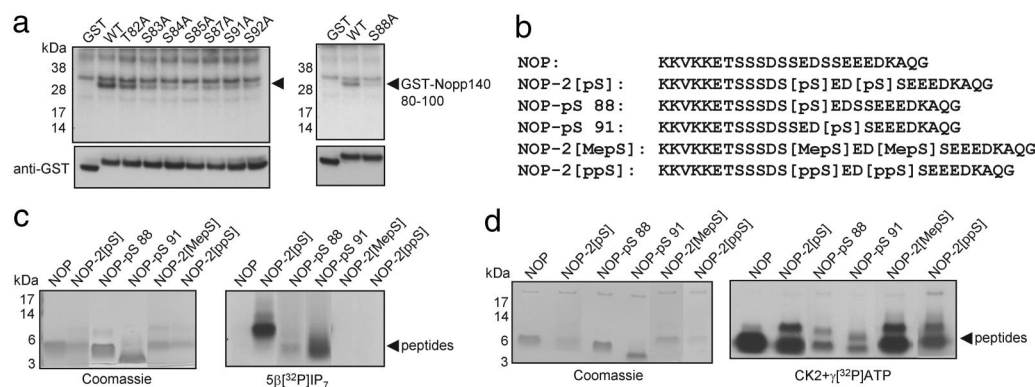


Fig. 2. IP₇ phosphorylates a phosphoserine residue to generate pyrophosphoserine. (a) Extracts from *S. cerevisiae* expressing GST-tagged mouse Nopp140 fragment (amino acids 80–100) WT sequence and indicated point mutants were phosphorylated by 5β[³²P]IP₇; autoradiography (Upper) and immunoblotting (Lower). (b) Sequences of individual peptides derived from Nopp140 (GenBank accession no. NP.941035). NOP, amino acids 76–100 of mouse Nopp140; NOP-2[pS], the same sequence as NOP except with phosphoserine at positions 88 and 91; NOP-pS 88 and NOP-pS 91, single phosphoserine residues at positions 88 and 91 respectively; NOP-2[MepS], methylphosphoserine at positions 88 and 91; NOP-2[ppS], pyrophosphoserine at positions 88 and 91. (c and d) The six synthetic peptides in b were incubated with 5β[³²P]IP₇ (c) or with CK2 and γ[³²P]ATP (d) and resolved by NuPAGE. Coomassie G250 staining (Left) and autoradiography (Right). In each peptide lane, there are multiple bands in the 3- to 9-kDa range, corresponding to peptide monomers and multimers, generated possibly because of electrostatic interactions between the positively charged lysines and negatively charged acidic and phosphate-containing residues (27). Peptide dimers were also observed by MALDI-TOF analysis (data not shown).

phosphorylation of the prephosphorylated but not the unphosphorylated Nopp140 peptide is consistent with pyrophosphorylation. This possibility is further supported by mass spectrometric analysis showing no phosphorylation by IP₇ of the unphosphorylated Nopp140 peptide but addition of one or two phosphates to the prephosphorylated peptides (SI Table 1). The maximum number of phosphates accepted by a peptide from IP₇ equals the number of preexisting phosphate groups on the peptide; single phosphoserine-containing peptides accept one phosphate from IP₇, whereas the peptide containing two phosphoserine residues accepts one or two phosphates.

The phosphorylated Nopp140 peptide might facilitate IP₇ phosphorylation of nearby serines or might be pyrophosphorylated. To distinguish those alternatives, we synthesized the same phosphopeptide in which the two phosphates have been methylated to prevent pyrophosphorylation (Fig. 2b). The methylated phosphopeptide is not phosphorylated by 5β[³²P]IP₇ (Fig. 2c and SI Table 1), although it can be phosphorylated by γ[³²P]ATP and CK2 (Fig. 2d).

The failure of the methylated phosphopeptide to be IP₇-phosphorylated strongly implies that IP₇ provides pyrophosphorylation. To further test this possibility, we synthesized the same Nopp140 peptide in which both serines 88 and 91 are pyrophosphorylated. This pyrophosphorylated peptide resists IP₇ phosphorylation, although it can be phosphorylated by CK2 (Fig. 2c and d and SI Table 1). This further supports the notion that IP₇ pyrophosphorylates its targets.

If IP₇ phosphorylates proteins on serines in the same fashion as ATP, then the properties of the phosphoserine bond should be the same with IP₇ and ATP phosphorylation. By contrast, the chemical properties of a pyrophosphorylated serine should differ from those of a conventional phosphoserine. We examined the acid sensitivity of the phospho-Nopp140 peptide after phosphorylation by γ[³²P]ATP and CK2 or 5β[³²P]IP₇ (Fig. 3a and b). Phosphorylation by 5β[³²P]IP₇ is more labile to treatment with 3 M HCl compared with phosphorylation by γ[³²P]ATP and CK2. Furthermore, we subjected the CK2 and ATP or IP₇-phosphorylated peptides to hydrolysis in the presence of 6 M HCl to release single amino acids. Hydrolysis liberates a prominent peak of [³²P]phosphoserine from the ATP-phosphorylated peptide but not from the IP₇-phosphorylated peptide (Fig. 3c–e). Thus, the pyrophosphate modification on serine is more

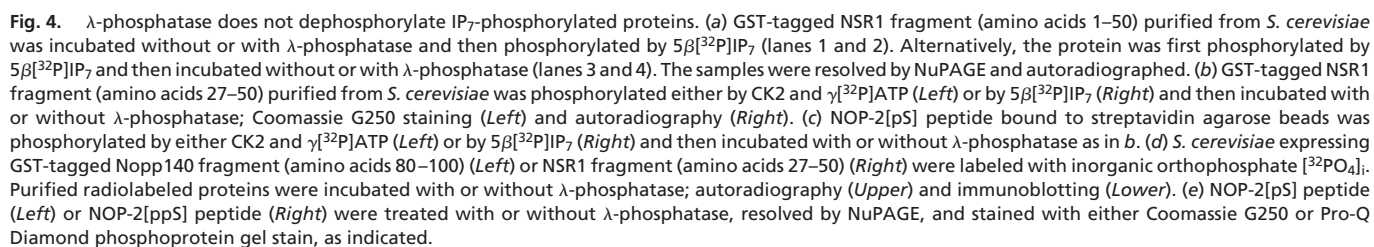
labile to acid than the conventional phosphoserine elicited by ATP-mediated phosphorylation.

In contrast to the greater lability to acid treatment, the IP₇-phosphorylated peptide is more stable to enzymatic dephosphorylation (Fig. 4a). As observed earlier (Fig. 1g), λ-phosphatase treatment before adding IP₇ prevents IP₇ phosphorylation of NSR1 (1–50). After IP₇ phosphorylation, however, the protein is completely resistant to λ-phosphatase. λ-Phosphatase greatly reduces phosphorylation of CK2 and ATP phosphorylated NSR1 (27–50) or Nopp140 peptide but fails to affect the IP₇-phosphorylated peptides (Fig. 4b and c). The same protein fragments are resistant to λ-phosphatase when purified from yeast labeled with inorganic orthophosphate [³²PO₄]_i (Fig. 4d), implying that these proteins exist within yeast cells in a pyrophosphorylated form.

These experiments suggest that the pyrophosphoserine bond is resistant to λ-phosphatase. We directly tested this possibility using the Nopp140 peptide containing pyrophosphorylated serines at position 88 and 91 compared with the same peptide with phosphorylated serines at these positions. λ-Phosphatase abolishes phosphorylation of the phospho-Nopp140 peptide but does not affect the pyrophosphorylated Nopp140 peptide (Fig. 4e and SI Table 2).

We also examined the sensitivity of the IP₇-phosphorylated NSR1 (27–50) fragment to other protein phosphatases. The following protein phosphatases are completely inactive: calcineurin, protein phosphatase-1, and alkaline phosphatase (SI Fig. 7a). In addition, the pyrophosphatase enzymes, thermostable inorganic pyrophosphatase and tobacco acid pyrophosphatase, fail to dephosphorylate the IP₇-phosphorylated peptide (SI Fig. 7a). Because these enzymes hydrolyze inorganic pyrophosphate (PPi), it is likely that they do not recognize pyrophosphate linked to a serine residue. We also studied DIPP (diphosphoinositol polyphosphate phosphohydrolase), an enzyme that Shears and associates (20, 21) have shown to physiologically dephosphorylate IP₇. As expected, pretreatment of the reaction mix containing IP₇ lowers phosphorylation, whereas treatment with DIPP after IP₇ phosphorylation has no effect (SI Fig. 7b).

In summary, we report that IP₇ pyrophosphorylates proteins. Evidence includes the requirement of prephosphorylation by ATP to prime proteins for IP₇ phosphorylation and the selective phosphorylation by IP₇ of synthetic phosphopeptides but not of



Therefore, it is likely that the principal known inositol pyrophosphates are able to pyrophosphorylate proteins.

Nonenzymatic pyrophosphorylation is thermodynamically feasible. Semiempirical calculations show that, whereas the free enthalpy (ΔH) of reaction for transfer of the β -phosphate from IP₇ to a simple primary alcohol is pH- and counterion-dependent, ΔH values at pH 6.8 can reach -38.3 kcal/mol (17). This strongly exothermic reaction equals or exceeds ΔH for phosphorylation by ATP. The high phosphorylation potential is attributable to the sterically and electronically crowded environment of the IP₇ pyrophosphate group. The phosphoserine to pyrophosphoserine conversion by IP₇ will require more advanced modeling, but two important clues emerge from the protein substrates for IP₇ that suggest how the 3D structures of the polypeptide may organize reagent and substrate to provide an entropic driving force to contribute to the overall free energy (ΔG) for the reaction. First, the polyserine tracts must have Asp or Glu residues present for pyrophosphorylation to occur. Second, the pyrophosphorylation shows an absolute requirement for divalent cations, with Mg²⁺ preferred (18). Both of these features are reminiscent of the prenyl diphosphate synthases, which feature DDXXD motifs that coordinate Mg²⁺, which in turn activates the pyrophosphate as a leaving group (22). For serine pyrophosphorylation, one can imagine IP₇ phosphates organized by H-bonds within a polySer tract, whereas the IP₇ pyrophosphate could be activated and targeted to a particular phosphoserine phosphate with the assistance of an Asp/Glu-chelated Mg²⁺.

What might be the physiologic role of pyrophosphorylation? Classic protein kinase-mediated phosphorylation modifies the conformation of proteins, enhancing or decreasing catalytic activity, determining protein localization, or altering protein-protein interactions. Presumably pyrophosphorylation also elicits such alterations in protein function. ATP-mediated phosphorylation is typically regulated by dephosphorylation, whereas pyrophosphorylated proteins are resistant to known protein phosphatases. This suggests that serine pyrophosphorylation may be a more stable modification within the cell, even though it is thermodynamically more unstable compared with phosphoserine. Such a conclusion is consistent with the fact that some of the best IP₇ targets, such as NSR1 and SRP40/Nopp140, exist endogenously as abundantly phosphorylated proteins (23–25). Although it is likely that pyrophosphorylation plays a central role in cellular processes modulated by inositol pyrophosphates, at this time, the biological significance of this modification remains unclear.

Materials and Methods

Synthesis of Inositol Pyrophosphate. The inositol pyrophosphate used throughout these studies was 5-PP-I(1,2,3,4,6)P₅ (referred to as IP₇). The synthesis and purification of radiolabeled 5 β [³²P]IP₇ was carried out as described earlier (18). By using these procedures, 5 β [³²P]IP₇ was obtained at a specific activity of 60 Ci/mmol (1 Ci = 37 GBq) and used at 1–2 μ Ci per reaction for protein phosphorylation. The synthesis of ³²P-labeled 4/6-PP-IP₅ and IP₈ is described in *SI Materials and Methods*. Unlabeled 5-PP-I(1,2,3,4,6)P₅ was prepared by total synthesis by using modifications of an earlier method (26). Full experimental details are provided in *SI Materials and Methods*.

Preparation of Recombinant Proteins. The expression and purification of all recombinant proteins are described in *SI Materials and Methods*.

Protein and Peptide Phosphorylation Assays. Cell lysates were obtained by resuspending yeast cells in ice-cold lysis buffer [20 mM Hepes (pH 6.8)/5 mM DTT/1 mM EGTA/1 mM EDTA/0.1% CHAPS, protease inhibitor mixture, and 200 mg/liter phenyl-

methanesulfonyl fluoride] and vortexing the sample in the presence of glass beads. The homogenates were centrifuged for 20 min at 15,000 \times g, and supernatants were used in the assay. In experiments where lysates were used directly for phosphorylation, protein extracts (10–20 μ g) were incubated in IP₇ phosphorylation buffer [25 mM Hepes (pH 7.4)/50 mM NaCl/6 mM MgCl₂/1 mM DTT] and 1 μ Ci 5 β [³²P]IP₇ for 15 min at 37°C. The reactions were heated at 95°C for 5 min in sample buffer before separation by NuPAGE (Invitrogen, Carlsbad, CA). The gels were either stained and dried, or transferred to a PVDF membrane. Radiolabeled proteins were detected by autoradiography. Immunoblotting using anti-Xpress (Invitrogen) or anti-GST (Sigma, St. Louis, MO) antibodies, was performed according to standard procedures.

GST- or hexahistidine-tagged proteins purified from *S. cerevisiae* were used directly for phosphorylation by 5 β [³²P]IP₇. The protein (1–2 μ g) was incubated in the presence of 5 β [³²P]IP₇, and the reaction was performed as described above. Where indicated, the purified proteins were preincubated with λ -phosphatase (400 units; New England Biolabs, Beverly, MA) at 30°C for 1 h, washed twice with PBS, and then used in a 5 β [³²P]IP₇ phosphorylation assay. GST fusion proteins purified from *E. coli*, were coupled to glutathione beads, and either incubated directly with 5 β [³²P]IP₇ as above or were first preincubated with 12 μ g of extract prepared from *S. cerevisiae* (strain DDY1810) at 30°C for 1 h in IP₇ phosphorylation buffer with or without 1 mM ATP or other nucleotides. Where indicated, the *S. cerevisiae* extract was incubated in boiling water for 5 min, cooled, and then used for the preincubation with purified protein. After incubation with yeast extract, the glutathione beads were washed twice with PBS and once with IP₇ phosphorylation buffer and used for phosphorylation by 5 β [³²P]IP₇ as described above. In some cases, proteins purified from *E. coli* were first preincubated with 250 units of CK2 (New England Biolabs) in the supplied CK2 reaction buffer, along with 1 mM ATP at 30°C for 1 h, and then washed and used for 5 β [³²P]IP₇ phosphorylation. NSR1 amino acids 27–50 purified from *E. coli* cells coexpressing human CK2A1 was used directly in 5 β [³²P]IP₇ phosphorylation assays.

Peptides were synthesized as described in *SI Materials and Methods*. Synthetic peptides were used for both CK2 and ATP or IP₇ mediated phosphorylation assays. Peptide (3–6 μ g) was incubated with 250 units of CK2 in CK2 phosphorylation buffer, 200 μ M ATP, and 1 μ Ci γ [³²P]ATP at 30°C for 15–30 min. For IP₇ phosphorylation, 3–6 μ g of peptide was added to IP₇ phosphorylation buffer and 1–2 μ Ci of 5 β [³²P]IP₇ at 37°C for 15 min, followed by incubation at 95°C for 5 min. The peptides were mixed with sample buffer and resolved on a 12% gel by using the NuPAGE system. The gels were stained by using Safe Stain (Invitrogen), dried, and used for autoradiography. Although peptide phosphorylation by IP₇ was routinely performed at 95°C to maximize the extent of phosphorylation, a time-dependent increase in phosphorylation is observed at 37°C (*SI Fig. 9*).

Acid-Sensitivity Assay. To determine the sensitivity of CK2 and ATP or IP₇-phosphorylated NOP-2[pS] peptide to treatment with acid, peptides were phosphorylated as described above and then incubated with either 1 M HCl at 50°C, with 3 M HCl at 25°C, or without any acid at 25°C, for 1 h. After incubation, the acid was neutralized with an appropriate volume of 10 M NaOH, and the reaction was mixed with sample buffer and subjected to NuPAGE. Phosphorylation was detected as described above. Peptide hydrolysis in the presence of 6 M HCl, followed by separation of amino acids by HPLC, was performed as described in *SI Materials and Methods*.

λ -Phosphatase-Sensitivity Assay. Purified GST fusion proteins or NOP-2[pS] peptide were phosphorylated by CK2 and γ [³²P]ATP or by 5 β [³²P]IP₇ as described above. After phosphorylation, the

biotinylated NOP-2[pS] peptide was bound to streptavidin agarose beads (Sigma) and washed with PBS. Protein or peptide was then incubated in the presence of λ -phosphatase (400 units; New England Biolabs) in supplied buffer according to the manufacturer's instructions at 30°C for 1 h, mixed with sample buffer, resolved by NuPAGE, and subjected to autoradiography. Expression and purification of $^{32}\text{[PO}_4\text{]}_i$ -labeled GST fusion proteins and their treatment with phosphatase is described in *SI Materials and Methods*.

The Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) was used to determine relative levels of phosphate on the NOP-2[pS] or NOP-2[ppS] peptides before or after treatment with λ -phosphatase. NOP-2[pS] peptide (6 μg) or NOP-2[ppS] peptide (24 μg)

were incubated with 400 units of λ -phosphatase for 1 h at 30°C and then resolved by NuPAGE and stained with Pro-Q Diamond stain according to the manufacturer's instructions.

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